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<b>13. ABSTRACT (Maximum 200 Words)</b> Retinoic acid (RA) has been used successfully in cancer prevention and therapy. RA exerts its biological effects through retinoic acid receptors (RARs, $\alpha$ , $\beta$ , $\gamma$ ) It has been reported that RAR $\beta$ plays an important role in mediating growth inhibitory actions of RA. The expression of RAR $\beta$ is lost in prostate cancer cell lines, PC-3, and DU-145, while transfection of RAR $\beta$ into PC-3 cells results in an increased sensitivity to growth inhibitory effects of RAR $\beta$ against. Despite the correlation between the level of RAR $\beta$ and the RA-associated growth inhibition, it remains unknown how RAR $\beta$ mediates the growth inhibitory effects of RA. This study used murine F9 wild type (Wt) and RAR $\beta$ knockout (F9 RAR $\beta^{-/-}$ ) cells as an experimental model to investigate the molecular mechanisms by which RAR $\beta$ mediates the growth inhibitory actions of RA. Our study demonstrated that p27, a cell cycle progression regulatory protein, is increased by RA in F9 Wt cells as compared to the F9 RAR $\beta^{-/-}$ cells. In addition, RA stabilizes the protein stability of p27. Considering the striking findings that transfection of RAR $\beta$ into the PC-3 cells results in an increased sensitivity to growth inhibition caused by RAR $\beta$ against, our study may lead to more efficient chemotherapy with retinoids.				
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## Introduction

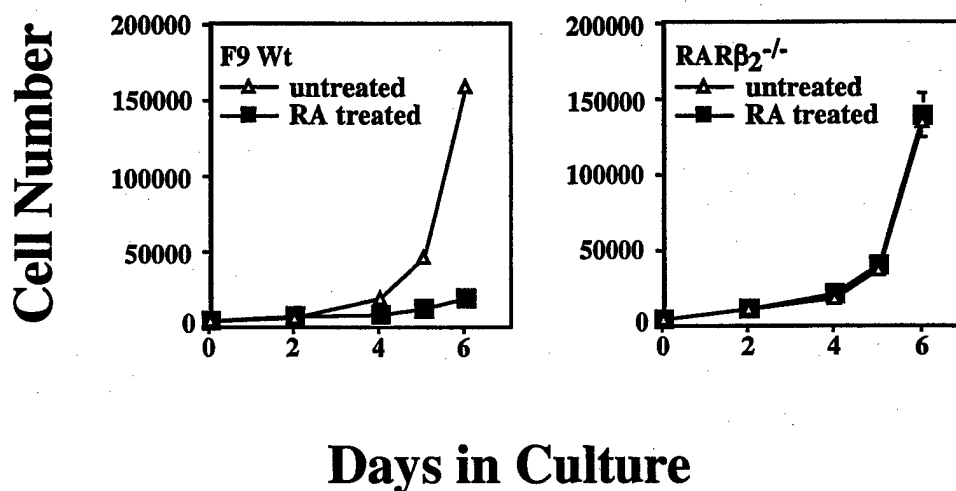
Prostate cancer is the most common cancer and the second leading cause of cancer deaths in males in the United States. Retinoids (retinol and its metabolites and derivatives) have been used in the prevention and treatment of some types of cancer. It has been shown that retinoic acid (RA), a biologically active form of retinol, is effective in inhibiting the cell growth and promoting differentiation of prostate cancer. It exerts its biological activities by binding to nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs). There are three RARs and three RXRs encoded by different genes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ). Each RAR and RXR gene encodes several protein isoforms, generated by different promoter usage or alternate splicing. The RAR $\beta_2$  isoform, the most abundant RAR $\beta$  isoform, is transcriptionally induced by RA in many cell types (1). A limitation to designing effective retinoid therapies in the treatment of prostate cancer is the lack of understanding of the molecular mechanisms that control retinoid-mediated growth inhibition and differentiation. It has been reported that prostate cancer cell lines PC-3 and DU-145 do not express RAR $\beta$ , while stable expression of RAR $\beta$  into the PC-3 cells results in an increased response to growth inhibition mediated by a RAR $\beta$  agonist and a hexafluoride vitamin D3 analog (2). There are data indicating that RAR $\beta$  plays an important role in mediating the growth inhibitory actions of RA. Conversely, the loss of RAR $\beta$  expression occurs during the process of carcinogenesis. Reduced expression of RAR $\beta$  is a common feature of premalignant lesions and carcinogenesis. (3-21). Malignant cells with decreased expression of RAR $\beta$  become resistant to RA treatment (15, 22, 23), whereas the up-regulation of RAR $\beta$  parallels RA-induced growth suppression in some tumor cells (24-26). In this study we studied the mechanisms by which RAR $\beta$  mediates the growth inhibitory actions of RA by using murine F9 wild type (F9 Wt) and F9 RAR $\beta_2$  knockout (F9 RAR $\beta_2^{-/-}$ ) cells as an experimental model.

## Body

We have previously shown that the F9 teratocarcinoma RAR $\beta_2$  knockout cell line exhibits no growth arrest in response to RA, whereas F9 Wt, F9 RAR $\alpha^{-/-}$  and F9 RAR $\gamma^{-/-}$  cell lines do growth arrest in response to RA. To examine the role of RAR $\beta_2$  in growth inhibition, we analyzed the cell cycle regulatory proteins affected by RA in F9 Wt and F9 RAR $\beta_2^{-/-}$  cells. Flow microfluorimetry analyses revealed that RA treatment of F9 Wt cells increased the percentage of cells in the G1/G0 phase of the cell cycle. In contrast, RA did not alter the cell cycle distribution profile of RAR $\beta_2^{-/-}$  cells. In F9 Wt cells, cyclin D1, D3 and cyclin E protein levels decreased, while cyclin D2 and p27 levels increased after RA treatment. Compared to the F9 Wt cells, the F9 RAR $\beta_2^{-/-}$  cells exhibited lower levels of cyclins D1, D2, D3, and E in the absence of RA, but did not exhibit further changes in the

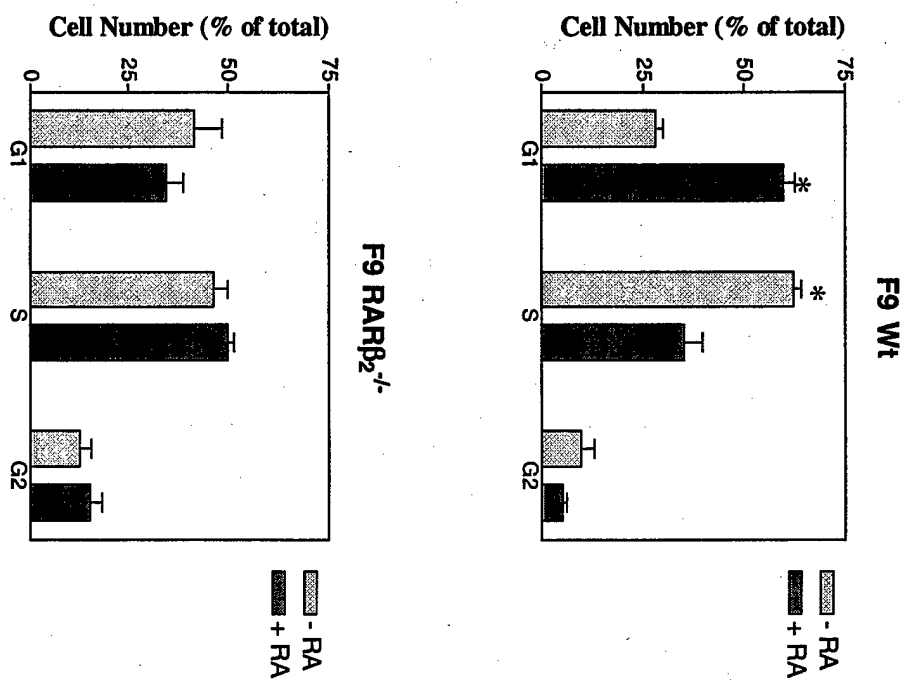
levels of these cell cycle regulators after RA addition. Since RA significantly increased the level of p27 protein (~ 24-fold) in F9 Wt as compared to the F9 RAR $\beta_2^{-/-}$  cells, we chose to study p27 in greater detail. The p27 protein plays a pivotal role in the regulation of the proliferation and differentiation of many cell types. Down-regulation of p27 has been observed in carcinogenesis and metastasis and the level of p27 has been used to evaluate cancer progression (27). The p27 mRNA level and the rate of p27 protein synthesis were increased in RA treated F9 Wt cells, but not in F9 RAR $\beta_2^{-/-}$  cells. Moreover, RA increased the half-life of p27 protein in F9 Wt cells. Reduced expression of RAR $\beta_2$  is associated with the process of carcinogenesis and RAR $\beta_2$  can mediate the growth arrest induced by RA in a variety of cancer cells. Using both genetic and molecular approaches, we have identified some of the molecular mechanisms, such as the elevation of p27, through which RAR $\beta_2$  mediates these growth inhibitory effects in F9 cells.

## RA Results in Cell Growth Arrest in F9 Wt but not in F9 RAR $\beta$ 2<sup>-/-</sup> Cells



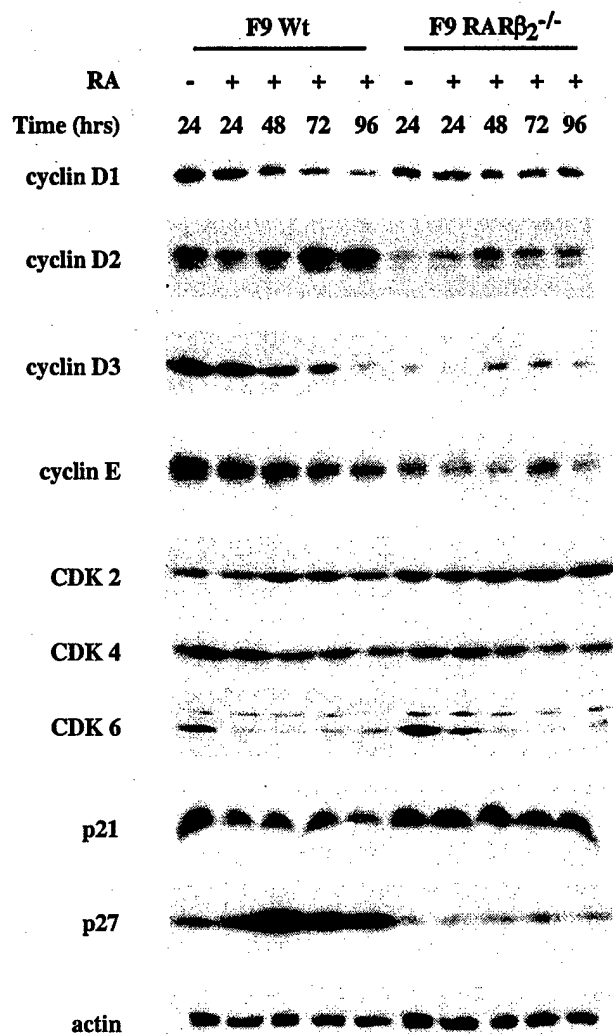
**Figure 1A. Analysis of the growth of F9 Wt and RAR $\beta$ 2<sup>-/-</sup> cells after treatment with 1  $\mu$ M RA. The cells were plated in duplicate wells at a density of 3000 cells/well. The cell numbers were counted on the indicated days. The experiment was performed three times with very similar results. The values represent the mean  $\pm$  S.D. of three independent experiments.**

# RA Increases the Percentage of Cells in G1 Phase in F9 Wt but not in F9 RAR $\beta_2$ <sup>-/-</sup> Cells



**Figure 1B.** Statistical analysis of the cell cycle distribution of F9 Wt and RAR $\beta_2$ <sup>-/-</sup> cells after treatment with 1  $\mu$ M RA for 96 hours. The values represent the mean  $\pm$  S.D. of three independent experiments. \*  $P < 0.05$ .

## RA Altered Cell Cycle Regulatory Proteins in F9 Wt and F9 RAR $\beta$ 2<sup>-/-</sup> Cells

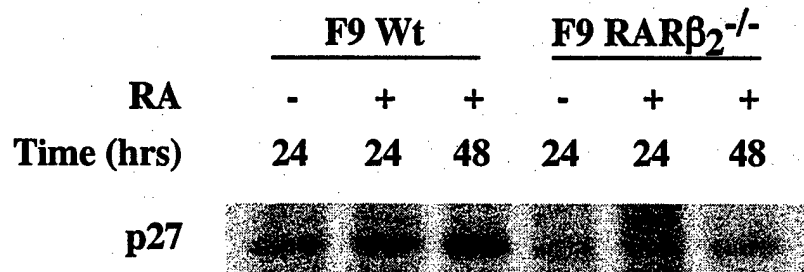


**Figure 2.** F9 Wt and RAR $\beta$ 2<sup>-/-</sup> cells were treated with 1  $\mu$ M RA for the times indicated. Total cell lysates were prepared and Western blot analysis was performed. The experiment was performed three times with each antibody with similar results. Actin was used as a loading control.

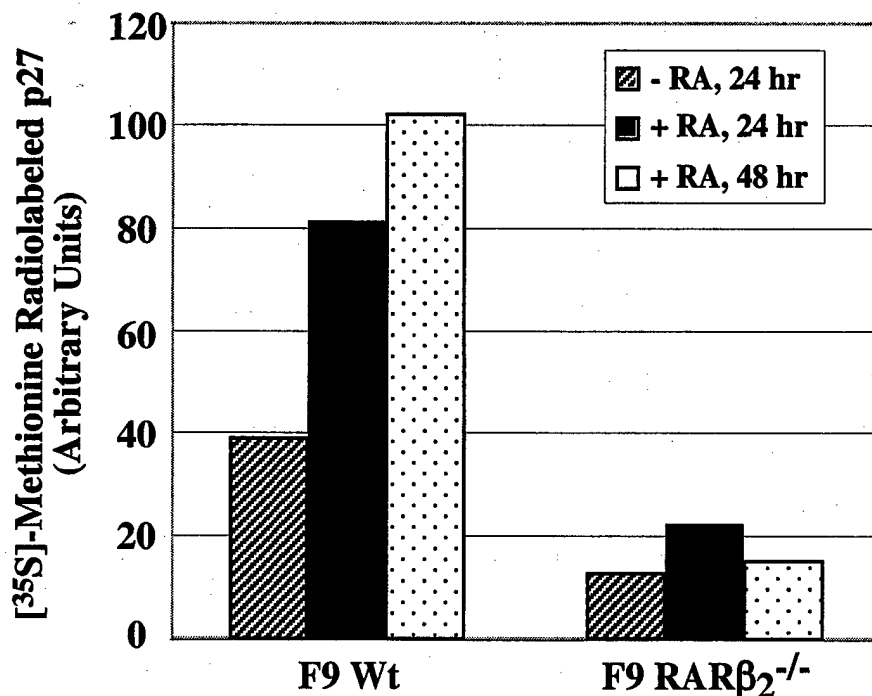


## RA Increases the Synthesis of p27 Protein in F9 Wt but not in F9 RAR $\beta$ <sub>2</sub><sup>-/-</sup> Cells

**A**



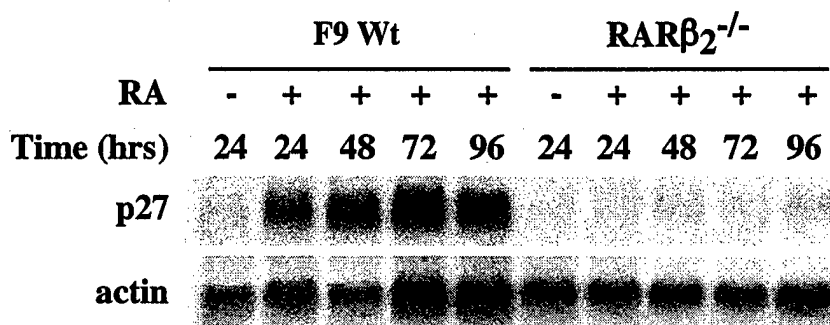
**B**



**Figure 3.** (A) F9 Wt and RAR $\beta$ <sub>2</sub><sup>-/-</sup> cells were treated with 1  $\mu$ M RA for the times indicated and then labeled with 50  $\mu$ Ci/ml [<sup>35</sup>S]-methionine for 30 minutes. Immunoprecipitation with anti-p27 antibody was performed. The radiolabeled protein precipitates were electrophoresed on a 10% SDS-polyacrylamide gel that was subjected to autoradiography. (B) The amount of signal in A was quantified by NIH Image. The experiment was performed three times with very similar results.

## RA Increases the Level of p27 mRNA in F9 Wt but not in F9 RAR $\beta$ <sub>2</sub><sup>-/-</sup> Cells

A



B

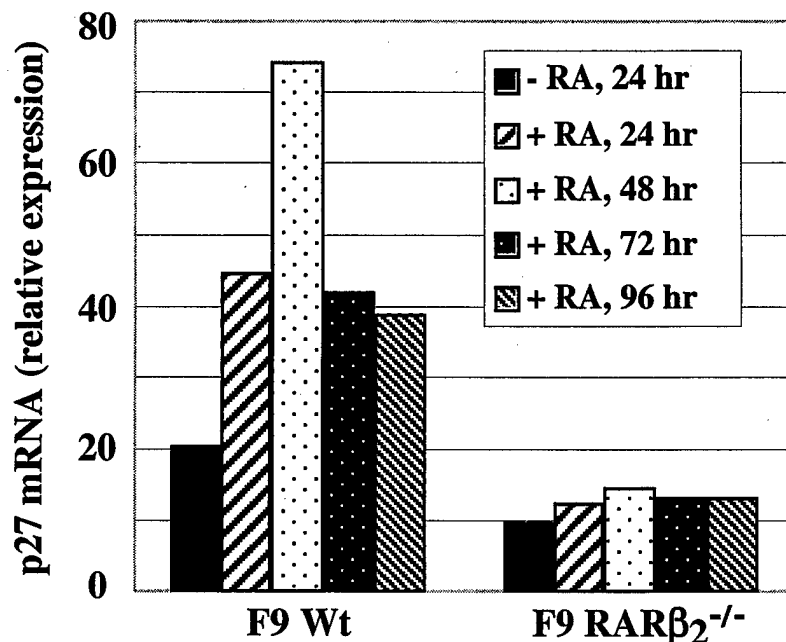
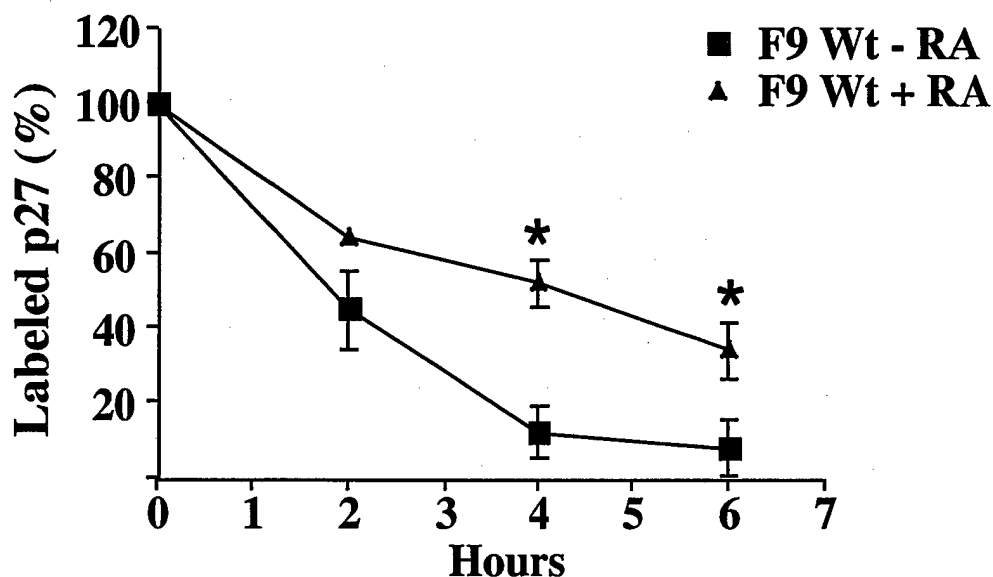


Figure 4. (A) F9 Wt and RAR $\beta$ <sub>2</sub><sup>-/-</sup> cells were treated with 1  $\mu$ M RA for 96 hours. Total cellular RNA was extracted and Northern blot analysis was used to detect the level of p27 mRNA. (B) The amount of signal in A was quantified by NIH Image. The relative expression level of p27 mRNA was depicted as the ratio of the density of p27 mRNA to actin mRNA for the same time point. This experiment was performed three times with very similar results.

## RA Stabilizes p27 Protein in F9 Wt Cells



**Figure 5.** F9 Wt cells were cultured in the absence or presence of 1  $\mu$ M RA for 48 hours, pulse-labeled with 100  $\mu$ Ci/ml [ $^{35}$ S]-methionine in the presence or absence of RA for 1 hour and chased for 6 hours. Immunoprecipitation with anti-p27 antibody was performed. The protein precipitates were electrophoresed on a 10% SDS-polyacrylamide gel that was subjected to autoradiography. The amount of p27 in the absence or presence of RA was analyzed with ImageQuant. The amount of p27 in the absence or presence of RA immediately after the 1 hour pulse labeling is set at 100. \*  $P < 0.05$ .

### **Key Research Accomplishments**

- Examined the effects of RA via RAR $\beta$  on the protein levels of several cell cycle regulatory proteins, one of which is p27.
- Investigated the effects of RA via RAR $\beta$  on the levels of p27 mRNA and protein.
- Determined the effects of RA via RAR $\beta$  on the stability of p27 protein.

### **Reportable Outcomes**

Delineated some of the molecular mechanisms by which RAR $\beta$  mediates the growth inhibitory effects of RA.

### **Conclusions**

The increase of p27 is associated with the growth inhibition induced by RA via RAR $\beta$  in F9 Wt cells. Considering the striking findings that stable transfection of RAR $\beta$  to PC-3 cells results in a sensitivity to growth inhibition caused by RAR $\beta$  against a vitamin D3 analog, these data may be of use in designing more efficient chemotherapy with retinoids.

### **Reference**

1. Mangelsdorf, D. J., Umesono, K., and Evans, R. M. The retinoid receptors. *In*: M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), *The Retinoids: Biology, Chemistry and Medicine*, 2nd edition, pp. 319-350. New York: Raven Press, 1994.
2. Campbell, M. J., Park, S., Uskokovic, M. R., Dawson, M. I., and Koeffler, H. P. Expression of retinoic acid receptor-beta sensitizes prostate cancer cells to growth inhibition mediated by combinations of retinoids and a 19-nor hexafluoride vitamin D3 analog. *Endocrinology*, 139: 1972-1980, 1998.
3. Hu, L., Crowe, D. L., Rheinwald, J. G., Chambon, P., and Gudas, L. J. Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res.*, 51: 3972-3981, 1991.
4. Xu, X. C., Ro, J. Y., Lee, J. S., Shin, D. M., Hong, W. K., and Lotan, R. Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues. *Cancer Res*, 54: 3580-3587, 1994.
5. Lotan, R., Xu, X. C., Lippman, S. M., Ro, J. Y., Lee, J. S., Lee, J. J., and Hong, W. K. Suppression of retinoic acid receptor-beta in premalignant oral lesions and its up-regulation by isotretinoin. *N Engl J Med*, 332: 1405-1410, 1995.

6. McGregor, F., Wagner, E., Felix, D., Soutar, D., Parkinson, K., and Harrison, P. R. Inappropriate retinoic acid receptor-beta expression in oral dysplasias: correlation with acquisition of the immortal phenotype. *Cancer Res*, 57: 3886-3889, 1997.
7. Sun, S. Y., Yue, P., Mao, L., Dawson, M. I., Shroot, B., Lamph, W. W., Heyman, R. A., Chandraratna, R. A., Shudo, K., Hong, W. K., and Lotan, R. Identification of receptor-selective retinoids that are potent inhibitors of the growth of human head and neck squamous cell carcinoma cells. *Clin Cancer Res*, 6: 1563-1573, 2000.
8. Chakravarti, N., Mathur, M., Bahadur, S., Shukla, N. K., Rochette-Egly, C., and Ralhan, R. Expression of RARalpha and RARbeta in human oral potentially malignant and neoplastic lesions. *Int J Cancer*, 91: 27-31, 2001.
9. Klaassen, I., Brakenhoff, R. H., Smeets, S. J., Snow, G. B., and Braakhuis, B. J. Expression of retinoic acid receptor gamma correlates with retinoic acid sensitivity and metabolism in head and neck squamous cell carcinoma cell lines. *Int J Cancer*, 92: 661-665, 2001.
10. Qiu, H., Zhang, W., El-Naggar, A. K., Lippman, S. M., Lin, P., Lotan, R., and Xu, X. C. Loss of retinoic acid receptor-beta expression is an early event during esophageal carcinogenesis. *Am J Pathol*, 155: 1519-1523, 1999.
11. Qiu, H., Lotan, R., Lippman, S. M., and Xu, X. C. Lack of correlation between expression of retinoic acid receptor-beta and loss of heterozygosity on chromosome band 3p24 in esophageal cancer. *Genes Chromosomes Cancer*, 28: 196-202, 2000.
12. Nervi, C., Vollberg, T. M., George, M. D., Zelent, A., Chambon, P., and Jetten, A. M. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. *Exp Cell Res*, 195: 163-170, 1991.
13. Zhang, X. K., Liu, Y., Lee, M. O., and Pfahl, M. A specific defect in the retinoic acid response associated with human lung cancer cell lines. *Cancer Res*, 54: 5663-5669, 1994.
14. Xu, X. C., Sozzi, G., Lee, J. S., Lee, J. J., Pastorino, U., Pilotti, S., Kurie, J. M., Hong, W. K., and Lotan, R. Suppression of retinoic acid receptor beta in non-small-cell lung cancer in vivo: implications for lung cancer development [see comments]. *J Natl Cancer Inst*, 89: 624-629, 1997.
15. Li, Y., Dawson, M. I., Agadir, A., Lee, M. O., Jong, L., Hobbs, P. D., and Zhang, X. K. Regulation of RAR beta expression by RAR- and RXR-selective retinoids in human lung cancer cell lines: effect on growth inhibition and apoptosis induction. *Int J Cancer*, 75: 88-95, 1998.
16. Picard, E., Seguin, C., Monhoven, N., Rochette-Egly, C., Siat, J., Borrelly, J., Martinet, Y., Martinet, N., and Vignaud, J. M. Expression of retinoid receptor

- genes and proteins in non-small-cell lung cancer. *J Natl Cancer Inst*, 91: 1059-1066, 1999.
17. Roman, S. D., Clarke, C. L., Hall, R. E., Alexander, I. E., and Sutherland, R. L. Expression and regulation of retinoic acid receptors in human breast cancer cells. *Cancer Res.*, 52: 2236-2242, 1992.
  18. Xu, X. C., Sneige, N., Liu, X., Nandagiri, R., Lee, J. J., Lukmanji, F., Hortobagyi, G., Lippman, S. M., Dhingra, K., and Lotan, R. Progressive decrease in nuclear retinoic acid receptor beta messenger RNA level during breast carcinogenesis. *Cancer Res*, 57: 4992-4996, 1997.
  19. Swisshelm, K., Ryan, K., Lee, X., Tsou, H. C., Peacocke, M., and Sager, R. Down-regulation of retinoic acid receptor beta in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. *Cell Growth Differ.*, 5: 133-141, 1994.
  20. Yang, Q., Mori, I., Shan, L., Nakamura, M., Nakamura, Y., Utsunomiya, H., Yoshimura, G., Suzuma, T., Tamaki, T., Umemura, T., Sakurai, T., and Kakudo, K. Biallelic inactivation of retinoic acid receptor beta2 gene by epigenetic change in breast cancer. *Am J Pathol*, 158: 299-303, 2001.
  21. Geisen, C., Denk, C., Gremm, B., Baust, C., Karger, A., Bollag, W., and Schwarz, E. High-level expression of the retinoic acid receptor beta gene in normal cells of the uterine cervix is regulated by the retinoic acid receptor alpha and is abnormally down-regulated in cervical carcinoma cells. *Cancer Res*, 57: 1460-1467, 1997.
  22. Berg, W. J., Nanus, D. M., Leung, A., Brown, K. T., Hutchinson, B., Mazumdar, M., Xu, X. C., Lotan, R., Reuter, V. E., and Motzer, R. J. Up-regulation of retinoic acid receptor beta expression in renal cancers in vivo correlates with response to 13-cis-retinoic acid and interferon-alpha-2a. *Clin Cancer Res*, 5: 1671-1675, 1999.
  23. Hayashi, K., Yokozaki, H., Naka, K., Yasui, W., Yajin, K., Lotan, R., and Tahara, E. Effect of 9-cis-retinoic acid on oral squamous cell carcinoma cell lines. *Cancer Lett*, 151: 199-208, 2000.
  24. Boyle, J. O., Langenfeld, J., Lonardo, F., Sekula, D., Reczek, P., Rusch, V., Dawson, M. I., and Dmitrovsky, E. Cyclin D1 proteolysis: a retinoid chemoprevention signal in normal, immortalized, and transformed human bronchial epithelial cells. *J Natl Cancer Inst*, 91: 373-379, 1999.
  25. Xu, X. C., Liu, X., Tahara, E., Lippman, S. M., and Lotan, R. Expression and up-regulation of retinoic acid receptor-beta is associated with retinoid sensitivity and colony formation in esophageal cancer cell lines. *Cancer Res*, 59: 2477-2483, 1999.

26. Lehmann, S., Paul, C., and Torma, H. Retinoid receptor expression and its correlation to retinoid sensitivity in non-M3 acute myeloid leukemia blast cells. *Clin Cancer Res*, 7: 367-373, 2001.
27. Sgambato, A., Cittadini, A., Faraglia, B., and Weinstein, I. B. Multiple functions of p27(Kip1) and its alterations in tumor cells: a review. *J Cell Physiol*, 183: 18-27, 2000.

In addition to what I previously reported, I studied the effects of retinoids and lecithin:retinol acyltransferase (LRAT) on the differentiation of human prostate cancer cells. LRAT is an enzyme involved in the metabolism of retinol to retinyl esters. It has been reported that the levels of LRAT and retinyl esters are reduced in some human cancers, such as prostate. The human prostate cancer cell line PC-3 was transfected with LRAT. The functional activity of LRAT in all the transfected cell lines was determined by HPLC (Figure 1). All the transfected cell lines took up and esterified retinol into retinyl esters, while PC-3 wild type cells did not. The PC-3 and PC-3/LRAT transfectant cells were treated with retinoic acid (RA) or retinol (ROL) for various times. RT-PCR was used to test the effects of retinoids and LRAT on several molecular markers of retinoid action, such as keratin 18 and Gbx2, in human prostate. Soft agar assays for tumor cell growth were also performed.

Our data showed that there were no obvious changes in the levels of the above molecular markers upon RA or ROL treatment in both PC-3 and PC-3/LRAT transfectant cells (Figure 2). These findings are important both in basic and clinical research. They indicate that retinyl esters are not crucial ligands for the regulation of the above genes in the carcinogenesis of human prostate. Our studies provide new information about retinoid effects on prostate cancer cells and provide a rationale for more efficient chemotherapy with retinoids.



# Retinol esterification in PC-3 Wt and PC-3/LRAT transfectants

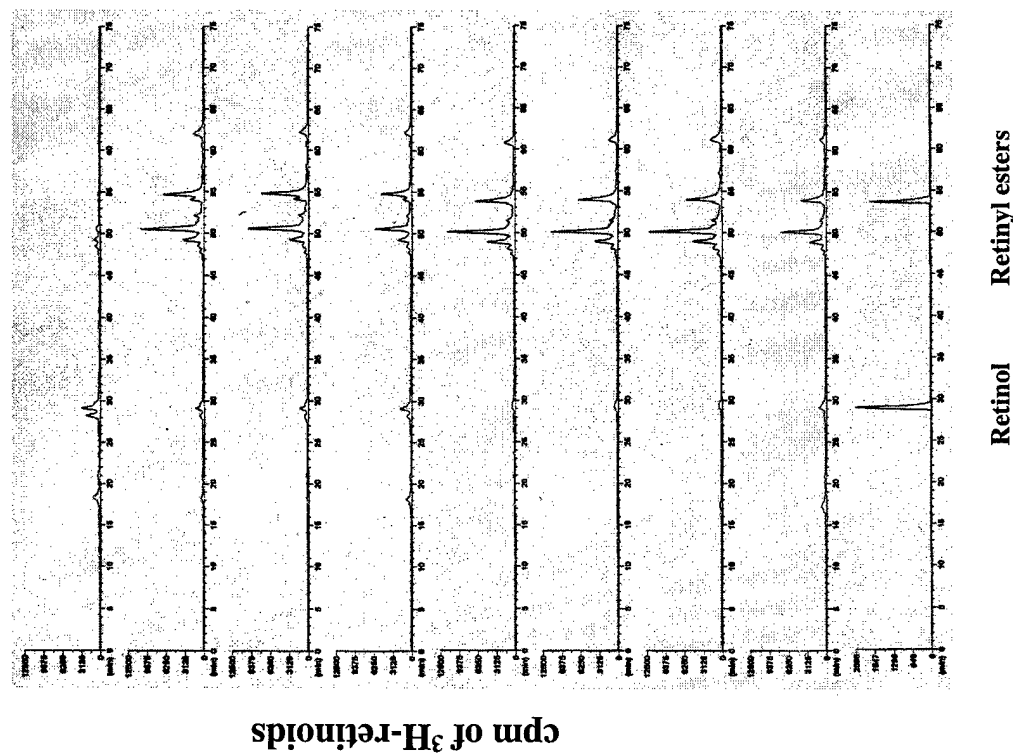
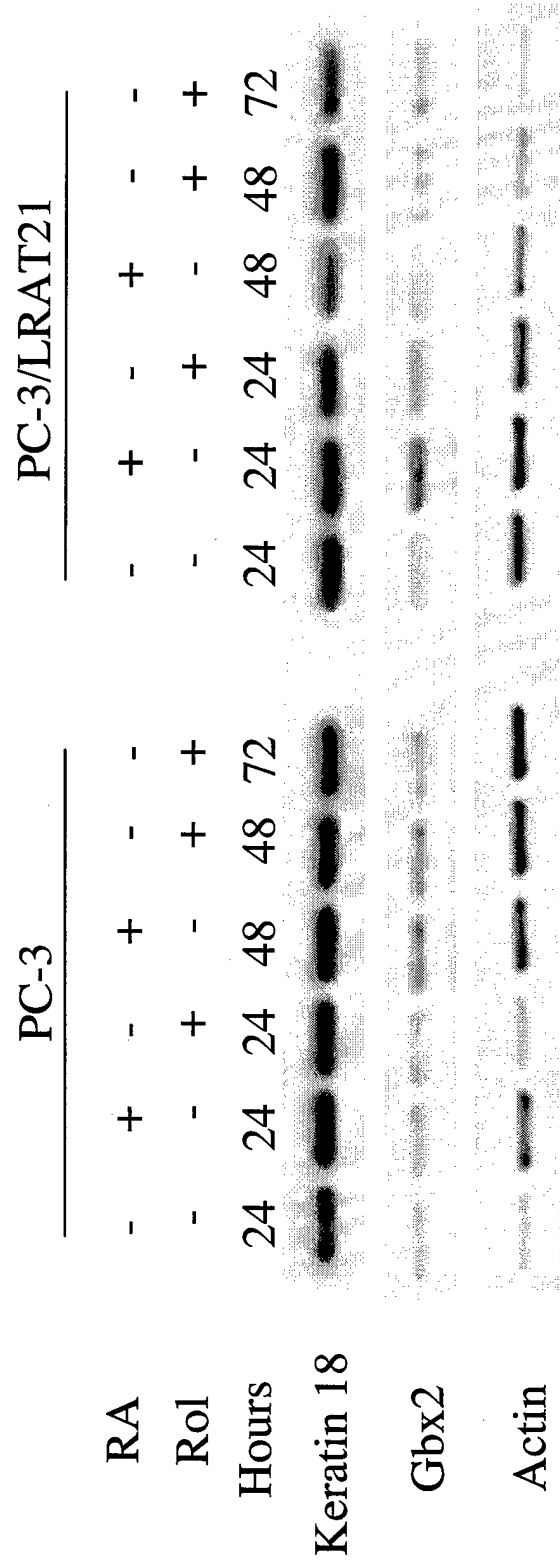


Figure 1. Retinol esterification in PC-3 wild type and PC-3/LRAT transfectants.



**Figure 2.** The effects of LRAT on the differentiation markers of human prostate.